

Transformations of Halogenated Aromatic Aldehydes by Metabolically Stable Anaerobic Enrichment Cultures

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Metabolically stable enrichment cultures of anaerobic bacteria obtained by elective enrichment of sediment samples from the Baltic Sea and Gulf of Bothnia have been used to study the oxidation and reduction of the aldehyde group of various halogenated aromatic aldehydes. During the transformation of 5- and 6-chlorovanillin, 6-bromovanillin, 3-chloro-4-hydroxybenzaldehyde, 3,5-dichloro-4-hydroxybenzaldehyde, and 3,5-dibromo-4-hydroxybenzaldehyde, it was shown that synthesis of the corresponding carboxylic acids, which were the principal metabolites, was invariably accompanied by partial reduction of the aldehyde to a hydroxymethyl group in yields of between 3 and 30%. Complete reduction to a methyl group was observed with some of the halogenated vanillins, but to an extremely limited extent with the halogenated 4-hydroxybenzaldehydes. One consortium produced both the hydroxymethyl and methyl compounds from both 5- and 6-chlorovanillin: it was therefore assumed that the methyl compound was the ultimate reduction product. On the basis of the kinetics of formation of the metabolites, it was concluded that the oxidation and reduction reactions were mechanistically related. In addition to these oxidations and reductions, dehalogenation was observed with one of the consortia. In contrast to the transformations of 5- and 6-chlorovanillin, which produced chlorinated methylcatechols, the corresponding compounds were not observed with 5- and 6-bromovanillin: the former was debrominated, forming 4-methylcatechol, whereas the latter produced 6-bromovanillyl alcohol without demethylation. Similarly, although 3-chloro-4-hydroxybenzaldehyde formed the chlorinated carboxylic acid and the benzyl alcohol, the 3-bromo compound was debrominated with formation of 4-hydroxybenzoic acid and, ultimately, phenol. On prolonged incubation, the halogenated carboxylic acids were generally decarboxylated, so that the final products from these substrates were halogenated catechols or phenols. Reductive processes of the type revealed in this study might therefore plausibly occur in the environment during anaerobic transformation of halogenated aromatic aldehydes containing hydroxyl and/or methoxyl groups.

Sediments in areas subject to industrial pollution frequently contain large amounts of organic carbon, so that they rapidly become highly anaerobic. The fate of xenobiotics contained in or deposited on these sediments is therefore determined largely by the outcome of anaerobic transformations.

We have been engaged in a study of the environmental fate of structurally diverse, low-molecular-weight chlorinated phenolic compounds formed during the production of bleached chemical pulp (7, 8). Among these compounds are chlorinated vanillins, whose presence we have confirmed in contaminated sediment samples collected near the site of discharge of bleachery effluent (M. Remberger, P.-Å. Hynning, and A. H. Neilson, *Environ. Toxicol. Chem.*, in press).

In a previous study (10) we showed that 5-chlorovanillin was metabolized to 3-chlorocatechol by a series of demethylation, oxidation, and decarboxylation reactions. We have now extended these investigations in two directions: (i) to examine the metabolism of 6-chlorovanillin, 3-chloro-4-hydroxybenzaldehyde, and 3,5-dichloro-4-hydroxybenzaldehyde, which are also components of bleachery effluents (7); and (ii) to include the reactions of the corresponding bromo compounds. We included the bromo compounds not only on account of their intrinsic interest, but also for the following reasons: (i) a number of aromatic bromo compounds are used in the synthesis of biocides applied initially to the terrestrial environment (2), and these may subse-

quently be introduced into the aquatic environment in the form of humus complexes which enter the sediment phase; and (ii) a number of aromatic bromo compounds used as flame retardants have been recovered from various sediment samples (18), where they are apparently highly stable. An investigation into the fate of structurally similar compounds under anaerobic conditions is therefore warranted.

In this communication, we are concerned primarily with reductive reactions involving the aldehyde group of halogenated aromatic aldehydes. We have shown that two sequential reductive processes accompany oxidation to the carboxylic acid: partial reduction to the benzyl alcohol and ultimate reduction to a methyl group. From the range of compounds examined here, we have attempted to elucidate the kinds of reactions which might be encountered in the anaerobic transformations of halogenated aromatic aldehydes.

MATERIALS AND METHODS

Microbiological methods. Procedures used previously (10) had to be modified slightly to take into account the low water solubility of most of the substrates (which made it impossible to prepare stock solutions) and to avoid precipitation at pH 3.5. The low-sulfate (0.2 g of Na_2SO_4 liter⁻¹) basal medium containing all the media components (including Casamino Acids [1.0 g liter⁻¹; Difco Laboratories, Detroit, Mich.], but excluding sulfide and bicarbonate) was prepared, the growth substrates were dissolved, and bicarbonate was added to give a concentration of 10 g liter⁻¹. The pH was adjusted to 6.7 before sterilization by filtration. Sulfide was subse-

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quently added to the medium in an anaerobe chamber to provide a final concentration of 0.36 g liter⁻¹.

All experiments were carried out at 22°C and with initial substrate concentrations of between 200 and 500 mg liter⁻¹. The consortia were obtained by elective enrichment from three sediment samples collected from the Gulf of Bothnia and the Baltic Sea (sediments 623, 685, and 688) as follows. Sediment 623 was used for an initial enrichment with 5-chlorovanillin, and when stable consortia had been attained, further adaptation was carried out with 5-bromovanillin, 3,5-dichloro-4-hydroxybenzaldehyde, or 3,5-dibromo-4-hydroxybenzaldehyde before beginning an experiment. In a similar way, sediment 685 was enriched initially with 3,5-dichloro-4-hydroxybenzoate before adaptation to 3,5-dichloro-4-hydroxybenzaldehyde or 3,5-dibromo-4-hydroxybenzaldehyde; and sediment 688 was enriched initially with 5-bromovanillin before adaptation to each of the following substrates: 5- and 6-chlorovanillin, 6-bromovanillin, 3-chloro-4-hydroxybenzaldehyde, 3-bromo-4-hydroxybenzaldehyde, 3,5-dichloro-4-hydroxybenzaldehyde, and 3,5-dibromo-4-hydroxybenzaldehyde.

Sealed glass ampoules were used for all the kinetic experiments (10), and control tubes lacking inoculum were incubated simultaneously to ensure that chemical transformation of the relatively reactive aldehydes had not occurred—especially reduction in the highly reducing medium. Resazurin was added to all ampoules to demonstrate the maintenance of anaerobic conditions throughout the sometimes lengthy experiments.

Analytical methods. (i) **GC methods.** The gas-chromatographic (GC) methods were the same as those previously described (10), with one minor modification necessitated by the fact that base-catalyzed acetylation of 4-hydroxybenzaldehyde derivatives with sodium acetate was not quantitative for all of the compounds. The following method was therefore adopted. The acetylation was carried out in two stages: in the first stage anhydrous sodium acetate was used as the base for 10 min at 75°C, and in the second stage pyridine (50 µl per sample) was used for a further 10 min. The acetylated sample was treated as previously described with the addition of a final wash with 4 ml of 0.5 M HCl to remove pyridine before GC analysis. The procedure used previously for esterification of carboxylic acids (10) successfully accomplished conversion of the benzyl alcohols to their O-methyl ethers. These derivatives were then acetylated as above. For comparison with authentic compounds, three independent derivatives of the phenolic compounds (O-acetate, O-ethyl ether, and O-trimethylsilyl ether) were prepared (10). For the phenolic benzyl alcohols, the same derivatives were used after conversion of the benzyl alcohols into their O-methyl ethers as described above. The GC retention times of these derivatives relative to tetrachloroguaiacol-O-acetate are given in Table 1. Except for phenol, the following temperature program was used: isothermal for 1 min at 150°C, followed by temperature increase at 2°C min⁻¹ to 265°C.

(ii) **GC-MS methods.** Spectra were run on a VG Masslab TRIO 2 mass spectrometer under the operating conditions described previously (12). The phenolic compounds were analyzed as their O-acetates, the carboxylic acids were analyzed as their methyl esters, and the benzyl alcohols were analyzed as their O-methyl ethers. For all of the compounds, the *m/z* values of the parent ions are given, together with the relative intensity of the isotopic patterns.

Substrates. 3-Chloro-4-hydroxybenzoic acid, 3,5-dichloro-4-hydroxybenzoic acid, 2-bromophenol, 2,6-dibromo-4-methylphenol, and the starting materials used for the syn-

TABLE 1. GC retention times of derivatives relative to that of tetrachloroguaiacol-O-acetate

Compound	Retention time for ^a :		
	O-Ace	O-TMS	O-Eth
Tetrachloroguaiacol	1.00	0.99	0.87
Phenols			
2-Chlorophenol	0.21	0.21	0.16
2-Bromo-4-methylphenol	0.33	0.44	0.31
2,6-Dichlorophenol	0.28	0.30	0.25
2,6-Dibromophenol	0.45	0.48	0.38
2,6-Dibromo-4-methylphenol	0.60	0.63	0.51
Catechols			
Catechol	0.31	0.27	0.19
4-Methylcatechol	0.41	0.33	0.23
3-Chlorocatechol	0.45	0.42	0.24
4-Chlorocatechol	0.48	0.42	0.29
3-Bromocatechol	0.56	0.53	0.40
3-Chloro-5-methylcatechol	0.58	0.61	0.40
4-Chloro-5-methylcatechol	0.63	0.52	0.30
3-Bromo-5-methylcatechol	0.72	0.63	0.58
Benzyl alcohols			
2-Chloro-4,5-dihydroxybenzyl alcohol	0.96	0.99	0.77
3-Chloro-4,5-dihydroxybenzyl alcohol	0.96	1.09	0.86
3-Chloro-4-hydroxybenzyl alcohol	0.46	0.44	0.44
2-Bromo-4-hydroxy-5-methoxybenzyl alcohol	0.92	0.83	0.81
3,5-Dichloro-4-hydroxybenzyl alcohol	0.63	0.67	0.55
3,5-Dibromo-4-hydroxybenzyl alcohol	0.95	0.99	0.84
Benzoic acids			
4-Hydroxybenzoic acid	0.42	0.43	0.38
3-Chloro-4-hydroxybenzoic acid	0.57	0.62	0.60
3,5-Dichloro-4-hydroxybenzoic acid	0.73	0.83	0.67
3,5-Dibromo-4-hydroxybenzoic acid	1.07	1.20	1.00
3-Chloro-4,5-dihydroxybenzoic acid	1.07	1.03	0.88
3-Bromo-4,5-dihydroxybenzoic acid	1.25	1.21	1.05
2-Chloro-4,5-dihydroxybenzoic acid	1.17	1.05	1.01
6-Bromo-3-methoxy-4-hydroxybenzoic acid	1.12	1.09	1.08
Tetrachloroguaiacol ^b	2.07	2.07	1.89
Phenol ^b	0.14	0.13	0.10

^a Abbreviations: O-Ace, O-acetate; O-TMS, O-trimethylsilyl ether; O-Eth, O-ethyl ether.

^b Temperature program: 100°C isothermal 1 min, increasing by 2°C min⁻¹ to 265°C.

thesis of the halogenated compounds described below were the purest available commercial products.

Purity of substrates. To assess the purity of the synthetic substrates, we examined the mass spectra of suitable derivatives; although all of these synthetic compounds had purities of >98% (GC-MS), they inevitably contained low concentrations of compounds with other degrees of halogenation, together with minor impurities in the aldehydes such as the corresponding carboxylic acids and benzyl alcohols. The presence of these low background levels has been taken into account: the concentrations reported from metabolic experiments exceeded these in all cases by a factor of at least 20.

Nomenclature of substrates and metabolites. To avoid possible confusion, some comment is necessary on the nomenclature we have used. Compounds which have methoxy substituents and which are related directly to vanillin are numbered as derivatives of vanillin, i.e., of 3-methoxy-

4-hydroxybenzaldehyde. All others are numbered to give precedence to the halogen atoms: for example, in this system, 6-chlorovanillyl alcohol would be termed 2-chloro-4-hydroxy-5-methoxybenzyl alcohol.

Synthesis of substrates and metabolites. 3,5-Dichloro-4-hydroxybenzaldehyde (m/z of the O-acetate $C_9H_6Cl_2O_3$, 232, 234, and 236 [in the ratio 10:6.5:1]) was prepared from 4-hydroxybenzaldehyde by chlorination with chlorine in refluxing chloroform for 4 h. The solvent was removed, and the residue was recrystallized from toluene. 3,5-Dibromo-4-hydroxybenzaldehyde (m/z of the O-acetate $C_9H_6Br_2O_3$, 320, 322, and 324 [in the ratio 1:2:1]) was prepared similarly by using bromine in chloroform and was filtered from the reaction mixture on cooling. It was crystallized from toluene and then from acetic acid.

3,5-Dibromo-4-hydroxybenzoic acid (m/z of the O-acetate methyl ester $C_{10}H_8Br_2O_4$, 350, 352, and 354 [in the ratio 1:2:1]) was prepared by oxidizing the aldehyde with alkaline silver oxide (13) and recrystallized from acetic acid.

3,5-Dichloro-4-hydroxybenzyl alcohol (m/z of the O-acetate O-methyl ether $C_{10}H_{10}Cl_2O_3$, 248, 250, and 252 [in the ratio 10:6.5:1]) and 3,5-dibromo-4-hydroxybenzyl alcohol (m/z of the O-acetate O-methyl ether $C_{10}H_{10}Br_2O_3$, 336, 338, and 340 [in the ratio 1:2:1]) were prepared by reducing the aldehydes with sodium borohydride in methanol for 3 h at room temperature. The products were crystallized twice from cyclohexane.

3-Chloro-4-hydroxybenzaldehyde (m/z of the O-acetate $C_9H_7ClO_3$, 198 and 200 [in the ratio 3:1]) and 3-bromo-4-hydroxybenzaldehyde (m/z of the O-acetate, $C_9H_7BrO_3$, 242 and 244 [in the ratio 1:1]) were prepared by Reimer-Tiemann reactions from 2-chlorophenol and 2-bromophenol, respectively. The products were crystallized from carbon tetrachloride.

3-Chloro-4-hydroxybenzyl alcohol (m/z of the O-acetate O-methyl ether $C_{10}H_{11}ClO_3$, 214 and 216 [in the ratio 3:1]) was prepared by lithium aluminum hydride reduction of the aldehyde in tetrahydrofuran at room temperature and crystallized from carbon tetrachloride.

The synthesis of 4-chlorocatechol (9) and 5-chlorovanillin and its metabolites (10) has already been described.

5-Bromovanillin (m/z of the O-acetate $C_{10}H_9BrO_4$, 272 and 274 [in the ratio 1:1]) was prepared by bromination of vanillin in refluxing acetic acid (0.5 h) with slightly more than 1 mol of bromine. The product was filtered from the cold solution and recrystallized from acetic acid.

5-Bromovanillic acid (crystallized from methanol; m/z of the O-acetate methyl ester $C_{11}H_{11}BrO_5$, 302 and 304 [in the ratio 1:1]) and 3-bromo-4,5-dihydroxybenzoic acid (crystallized from xylene; m/z of the O-acetate methyl ester $C_{12}H_{11}BrO_6$, 330 and 332 [in the ratio 1:1]) were prepared by methods used for the chloro analogs (10). 3-Bromo-4,5-dihydroxybenzaldehyde (crystallized from acetic acid; m/z of the di-O-acetate $C_{11}H_9BrO_5$, 300 and 302 [in the ratio 1:1]) was prepared by demethylation of 5-bromovanillin with $BBr_3 \cdot (CH_3)_2S$ in refluxing 1,2-dichloroethane for 1 h (19).

6-Chlorovanillin (m/z of the O-acetate $C_{10}H_9ClO_4$, 228 and 230 [in the ratio 3:1]) and 6-bromovanillin (m/z of the O-acetate $C_{10}H_9BrO_4$, 272 and 274 [in the ratio 1:1]) were prepared by chlorination or bromination of vanillin tri-O-acetate in acetic acid containing sodium acetate to prevent the solution becoming acidic (15). The products were crystallized from ethanol and hydrolyzed with methanolic KOH, the free phenols were liberated by addition of H_2SO_4 , the mixture was filtered, and the solid residue was extracted

several times with boiling methanol. The solvent was removed, and the products were crystallized from acetic acid.

3-Chloro-4,5-dihydroxybenzyl alcohol and 2-chloro-4,5-dihydroxybenzyl alcohol could not be prepared in a pure state. Material of sufficient purity for identification purposes was, however, obtained by the following procedure. 5- and 6-chlorovanillin were reduced to their chlorovanillyl alcohols with lithium aluminum hydride in tetrahydrofuran at room temperature and crystallized from carbon tetrachloride and ethyl acetate, respectively (m/z of the O-acetate O-methyl ethers $C_{11}H_{13}ClO_4$, 244 and 246 [in the ratio 3:1]). The chlorovanillyl alcohols were demethylated with $BBr_3 \cdot (CH_3)_2S$ in boiling 1,2-dichloroethane (19) to yield the desired benzyl alcohols (m/z of the di-O-acetate O-methyl ethers, $C_{12}H_{13}ClO_5$, 272 and 274 [in the ratio 3:1]). The same products could also be obtained, albeit in poor yield, by the reverse procedure: 5- or 6-chlorovanillin was demethylated with $BBr_3 \cdot (CH_3)_2S$ in refluxing 1,2-dichloroethane to give the 3- or 2-chloro-4,5-dihydroxybenzaldehyde (crystallized from acetic acid; m/z of the di-O-acetates $C_{11}H_9ClO_5$, 256 and 258 [in the ratio 3:1]), which were then reduced as above with lithium aluminum hydride.

6-Chlorovanillic acid (crystallized from water; m/z of the methyl ester O-acetate $C_{11}H_{11}ClO_5$, 258 and 260 [in the ratio 3:1]) and 2-chloro-4,5-dihydroxybenzoic acid (crystallized from ethyl acetate-hexane; m/z of the methyl ester di-O-acetate $C_{12}H_{11}ClO_6$, 286 and 288 [in the ratio 3:1]) were prepared by procedures analogous to those used for the corresponding metabolites from 5-chlorovanillin (10), except that the de-O-methylations were carried out with $BBr_3 \cdot (CH_3)_2S$ in refluxing 1,2-dichloroethane (19). 6-Bromovanillic acid (crystallized from water; m/z of the methyl ester O-acetate $C_{11}H_{11}BrO_5$, 302 and 304 [in the ratio 1:1]) was prepared from 6-bromovanillin by the method used for 6-chlorovanillic acid.

2-Bromo-4-hydroxy-5-methoxybenzyl alcohol (6-bromovanillyl alcohol) was prepared from 6-bromovanillin by reduction with lithium aluminum hydride in tetrahydrofuran as described above and crystallized from ethyl acetate (m/z of the O-acetate O-methyl ether $C_{11}H_{13}BrO_4$, 288 and 290 [in the ratio 1:1]).

3-Bromosalicylaldehyde was prepared by a Reimer-Tiemann reaction from 2-bromophenol (crystallized from carbon tetrachloride; m/z of the O-acetate $C_9H_7BrO_3$, 242 and 244 [in the ratio 1:1]) and oxidized by the modified Dakin method (see reference 4) to 3-bromocatechol (obtained as an amber-colored oil which could not be crystallized; m/z of the di-O-acetate $C_{10}H_9BrO_4$, 272 and 274 [in the ratio 1:1]).

2-Bromo-4-methylphenol was prepared by brominating 4-methylphenol with bromine (1.1 mol) in carbon disulfide at 0°C. The product was a liquid (m/z of the O-acetate $C_9H_9BrO_2$, 228 and 230 [in the ratio 1:1]).

4-Chloro-5-methylcatechol (m/z of the O-acetate $C_{11}H_{11}ClO_4$, 242 and 244 [in the ratio 3:1]) was prepared from 4-methylcatechol by chlorination with sulfuric chloride in diethyl ether. It was obtained as long needles after crystallization several times from hexane-toluene. The corresponding bromo compound (m/z of the O-acetate $C_{11}H_{11}BrO_4$, 286 and 288 [in the ratio 1:1]) was prepared by bromination with bromine in carbon disulfide at room temperature. The crystalline solid obtained by evaporation of the solvent was recrystallized first from toluene and then several times from hexane.

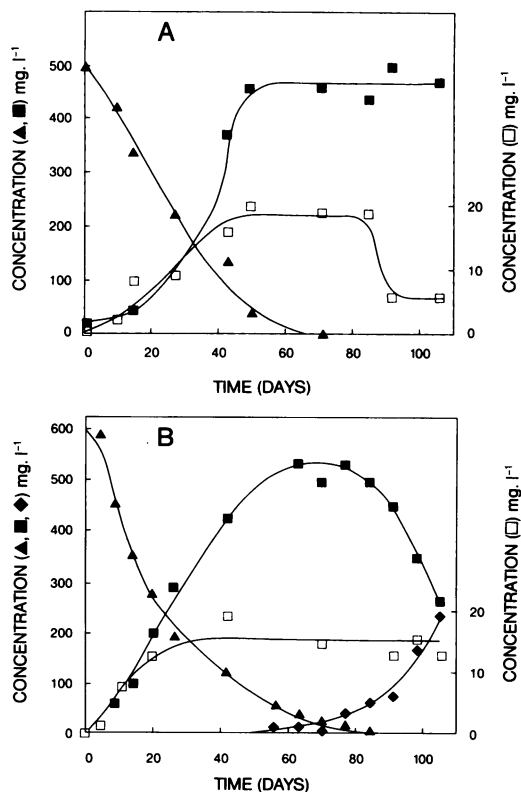


FIG. 1. Kinetics of the transformation by consortium 688 of (A) 3,5-dibromo-4-hydroxybenzaldehyde and (B) 3,5-dichloro-4-hydroxybenzaldehyde. Panel A symbols: ▲, 3,5-dibromo-4-hydroxybenzaldehyde; ■, 3,5-dibromo-4-hydroxybenzoic acid; □, 3,5-dibromo-4-hydroxybenzyl alcohol. Panel B symbols: ▲, 3,5-dichloro-4-hydroxybenzaldehyde; ■, 3,5-dichloro-4-hydroxybenzoic acid; □, 3,5-dichloro-4-hydroxybenzyl alcohol; ◆, 2,6-dichlorophenol.

RESULTS

Examination of control ampoules (without inocula) incubated during the experiments showed that the substrates were stable under the experimental conditions and that there was no increase in the concentrations of the carboxylic acids and benzyl alcohols (which existed as minor impurities in some of the substrates). For the halogenated vanillins, the concentrations of these metabolites were below the limits of detection. For 3,5-dichloro-4-hydroxybenzaldehyde, the concentration of the carboxylic acid was $<5 \text{ mg liter}^{-1}$ and the benzyl alcohol was undetectable; for 3,5-dibromo-4-hydroxybenzaldehyde, the corresponding concentrations were 8 and 1 mg liter^{-1} , respectively.

The kinetics of transformation of 3,5-dibromo-4-hydroxybenzaldehyde and 3,5-dichloro-4-hydroxybenzaldehyde by consortium 688 are shown in Fig. 1A and B. Identification of the benzyl alcohols was based on GC and GC-MS comparison with authentic compounds (Fig. 2A and B), and identification of the acids was based on GC comparison with authentic compounds. Low concentrations of the fully reduced compounds 2,6-dibromo-4-methylphenol, 2-bromo-4-methylphenol, and 2,6-dichloro-4-methylphenol were also formed. GC-MS comparison of the first with the authentic compound is shown in Fig. 2C: a sample of the corresponding chloro compound was not readily available, so that identification of the metabolite was based on the similarity of

the mass spectrum of the O-acetate ($\text{C}_9\text{H}_8\text{Cl}_2\text{O}_2$; m/z 218, 220, and 222 [in the ratio 10:6.5:1]) to that of the dibromo compound. 2-Bromo-4-methylphenol was identified by comparison of the mass spectrum of the O-acetate ($\text{C}_9\text{H}_9\text{BrO}_2$; m/z 228 and 230 [in the ratio 1:1]) with that of an authentic sample. The concentrations of the dibromomethylphenol and the dichloromethylphenol were ca. 10% and ca. 0.6%, respectively, of that of the corresponding benzyl alcohol. Although 3,5-dibromo-4-hydroxybenzyl alcohol was not completely stable under the experimental conditions, we have not determined its ultimate fate: the low concentration of the fully reduced 2,6-dibromo-4-methylphenol, which was identified by GC-MS, clearly, however, does not account for the observed diminution in the concentration of the benzyl alcohol. The carboxylic acids were ultimately decarboxylated to 2,6-dibromophenol and 2,6-dichlorophenol, which were identified by GC comparison with authentic compounds.

Consortia 623 and 685 also totally metabolized the 3,5-dihalogeno-4-hydroxybenzaldehydes, with the production of the carboxylic acids and benzyl alcohols whose concentrations were ca. 2 to 5% of those of the carboxylic acids for the chloro compound and ca. 8 to 10% for the bromo compound.

3-Chloro-4-hydroxybenzaldehyde was totally metabolized by consortium 688 first to 3-chloro-4-hydroxybenzyl alcohol and then to 3-chloro-4-hydroxybenzoic acid. The carboxylic acid was then partly decarboxylated (ca 2%) to 2-chlorophenol, which was identified by GC comparison with a reference sample. The ratio of the benzyl alcohol to the carboxylic acid was ca. 1:5. GC-MS comparison of the benzyl alcohol with an authentic compound is given in Fig. 2D. By contrast, the same consortium debrominated 3-bromo-4-hydroxybenzaldehyde to produce 4-hydroxybenzoic acid, which was quantitatively decarboxylated to phenol. These metabolites were identified by GC comparison with authentic compounds.

The kinetics of transformation of 5-chlorovanillin and 5-bromovanillin by consortium 623 are shown in Fig. 3A and B. The identity of the principal metabolites from 5-chlorovanillin had already been confirmed in a previous study (10) with a different consortium and with addition of tetrachloroguaiacol as cosubstrate. GC-MS comparison of the chloro- and bromomethylcatechols was carried out by using the more readily available 4-halogeno-5-methyl isomers (Fig. 4A and B). Although the mass spectra of the two isomers were identical except for minor differences in the intensities of the parent ions, the GC retention times were not. The mass spectra of the O-ethyl ethers (m/z $\text{C}_{11}\text{H}_{15}\text{ClO}_2$, 214 and 216 [in the ratio 3:1], and $\text{C}_{11}\text{H}_{15}\text{BrO}_2$, 258 and 260 [in the ratio 1:1]) and of the O-trimethylsilyl ethers (m/z $\text{C}_{13}\text{H}_{23}\text{ClO}_2\text{Si}_2$, 302 and 304 [in the ratio 3:1], and $\text{C}_{13}\text{H}_{23}\text{BrO}_2\text{Si}_2$, 346 and 348 [in the ratio 1:1]) were entirely consistent with those expected for monohalogenated methylcatechols. In the absence of evidence for migration of ring substituents, we therefore drew the reasonable conclusion that the metabolites were the 3-halogeno-5-methyl isomers, and for quantification that the GC responses were the same as those of the 4-halogeno-5-methylcatechols. The other metabolites from 5-bromovanillin were identified by GC-MS comparison with authentic reference compounds (Fig. 4C and D).

Kinetic experiments were also carried out with consortium 688, which metabolized both 5-chlorovanillin and 5-bromovanillin completely (data not shown). The principal ultimate metabolite from 5-chlorovanillin (ca. 95%) was 3-chlorocatechol, the remainder consisting principally of 3-chloro-4,5-dihydroxybenzyl alcohol, 3-chloro-5-methylca-

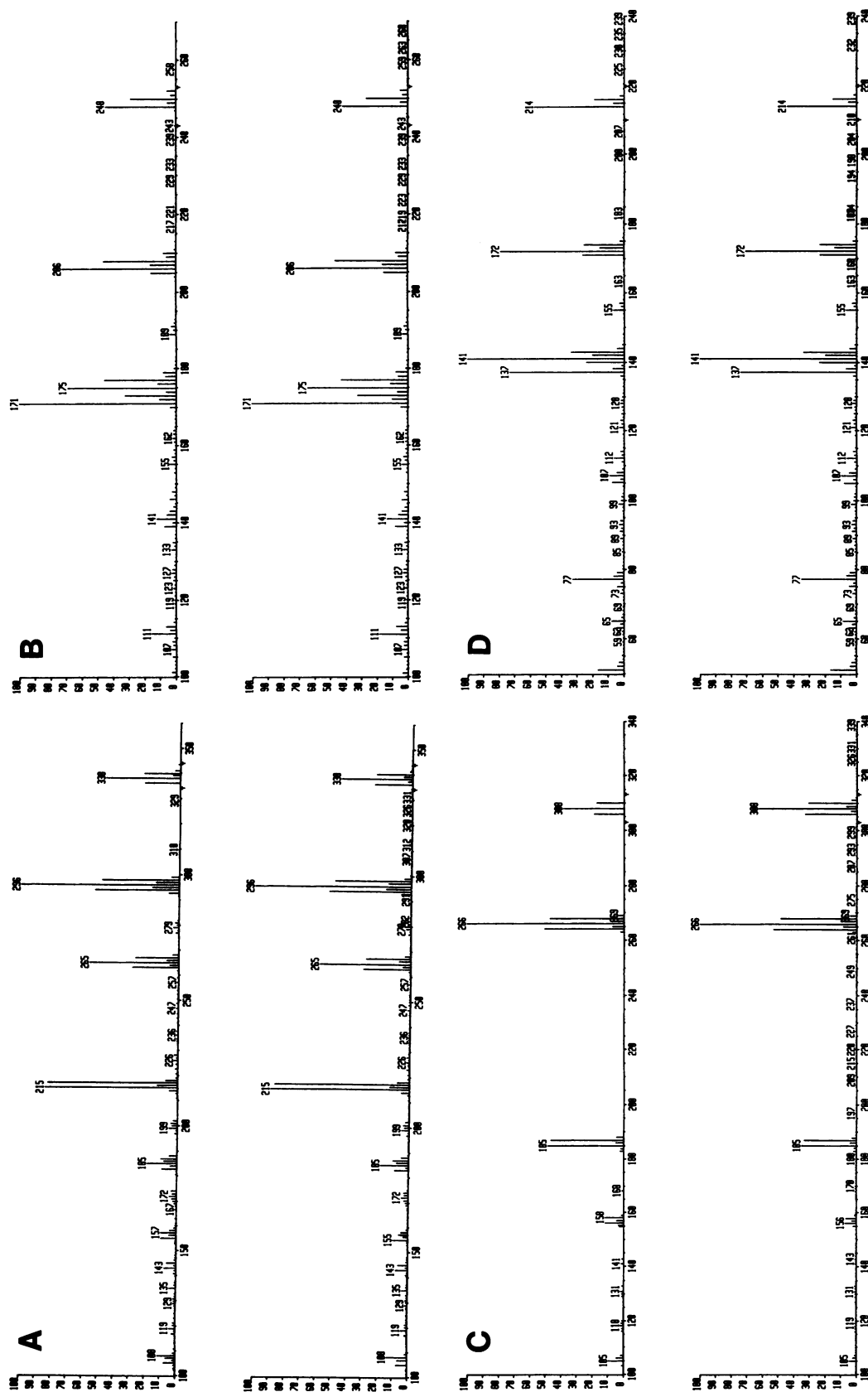


FIG. 2. MS comparison of the derivatized hydroxymethyl compounds (benzyl alcohols) produced by consortium 688 from (A) 3,5-dibromo-4-hydroxybenzaldehyde and (B) 3,5-dichloro-4-hydroxybenzaldehyde with standard compounds. The mass spectrum of the corresponding methyl compound produced from panel A is shown in panel C and the benzyl alcohol from 3-chloro-4-hydroxybenzaldehyde is shown in panel D. Upper panels refer to the metabolites, and lower panels refer to the standard compounds.

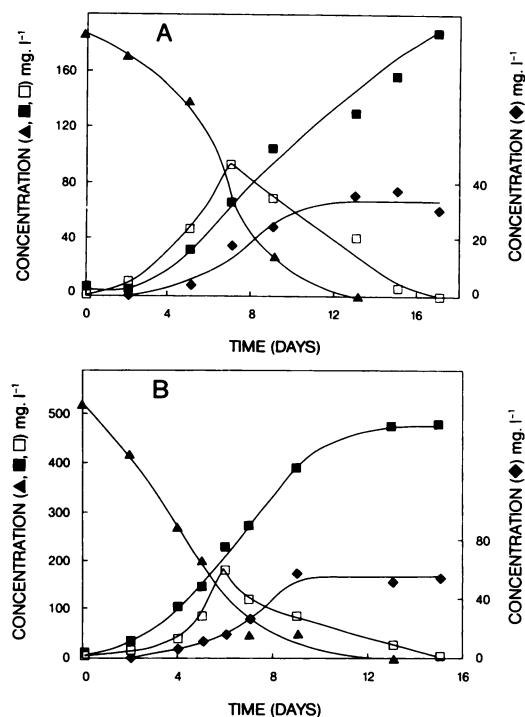


FIG. 3. Kinetics of transformation by consortium 623 of (A) 5-chlorovanillin and (B) 5-bromovanillin. Panel A symbols: \blacktriangle , 5-chlorovanillin; \square , 3-chloro-4,5-dihydroxybenzaldehyde; \blacksquare , 3-chloro-4,5-dihydroxybenzoic acid; \blacklozenge , 3-chloro-5-methylcatechol. Panel B symbols: \blacktriangle , 5-bromovanillin; \square , 3-bromo-4,5-dihydroxybenzaldehyde; \blacksquare , 3-bromo-4,5-dihydroxybenzoic acid; \blacklozenge , 3-bromo-5-methylcatechol.

techol, and 3-chloro-4,5-dihydroxybenzoic acid in the ratio of ca. 2.5:1.5:1. The benzyl alcohol was identified by GC-MS comparison with a reference sample (Fig. 5A). The same consortium debrominated 5-bromovanillin and decarboxylated the carboxylic acids, so that the final products were 3-bromocatechol, catechol, 4-methylcatechol, and 3-bromo-5-methylcatechol in the ratio of ca. 75:60:2:1. GC-MS comparison of the debrominated metabolites with authentic compounds is given in Fig. 5B and C.

6-Chlorovanillin was transformed by consortium 688 in a manner similar to that of 5-chlorovanillin, except that the 2-chloro-4,5-dihydroxybenzoic acid, which was the major metabolite (ca. 95%), was only partly decarboxylated to 4-chlorocatechol: the reduced products 2-chloro-4,5-dihydroxybenzyl alcohol, 4-chlorocatechol, and 4-chloro-5-methylcatechol were formed in the ratio of ca. 4:2:1. These metabolites were identified by GC-MS comparison with authentic compounds (Fig. 6A to C). By contrast, 6-bromovanillin was not demethylated and was metabolized only to the extent of ca. 6%: 6-bromovanillic acid and 2-bromo-4-hydroxy-5-methoxybenzyl alcohol (6-bromovanillyl alcohol), which were the sole metabolites, were formed in approximately equal amounts. The benzyl alcohol was identified by GC and GC-MS (Fig. 6D) comparison with the authentic compound.

DISCUSSION

Before kinetic experiments were undertaken, metabolically stable enrichment cultures were obtained as described previously (10): we have used the term consortium for these,

even though we have identified neither the nature nor the extent of the interaction between the components. Although the observed transformations were clearly brought about by the anaerobic bacteria in the consortia, however, it is difficult, for the following reasons, to precisely define the metabolic situation occurring in these experiments.

GC-MS examination of derivatized samples from inoculated samples showed the presence of substantial concentrations of 4-methylphenol, phenylacetic acid, and 4-hydroxyphenylacetic acid, which are putative metabolites of components of the Casamino Acids. The amino acids could therefore have played a significant metabolic role in at least two ways: (i) they may have acted as growth substrates for the cultures, so that the observed transformations might be more accurately described as taking place during concurrent metabolism (11); and (ii) the Casamino Acids might participate in redox processes analogous to the Stickland reaction (5). We believe, however, that the halogenated compounds added as substrates played a significant role: growth with those substrates containing methoxy groups which were metabolized (5-chlorovanillin, 6-chlorovanillin, and 5-bromovanillin) was always greater than with the nonmethoxylated halogenated benzaldehydes or 6-bromovanillin, which was not demethylated. This is consistent with the demonstration that in the acetogenic *Acetobacterium woodii*, successively increased growth was associated with aromatic carboxylic acids having larger numbers of methoxy groups (3). Further speculation is clearly premature in the absence of experimental details, particularly in view of the fact that the consortia comprise several kinds of bacteria whose metabolic capacity is currently unresolved.

The GC and GC-MS data unambiguously support the structures assigned to the benzyl alcohols and methylcatechols. Since this reductive capability was demonstrated for consortia obtained by enrichment of sediment samples collected from two geographically separated areas, we suggest that it was probably widespread in anaerobic sediments in the Baltic Sea and Gulf of Bothnia.

Although fully reduced methyl compounds were formed only in extremely low concentrations from the 3,5-dihalo-4-hydroxybenzaldehydes, isolation of both the benzyl alcohol and the methyl compound from 5- and 6-chlorovanillin by one of the consortia (consortium 688) strongly supported the mechanistically plausible view that the benzyl alcohol was the precursor of the fully reduced methyl compound.

The reduced metabolites were formed, together with the corresponding carboxylic acids, which were the major detectable primary metabolites except for 3-bromo-4-hydroxybenzaldehyde. Thus, there was not a stoichiometric relation between the oxidation and reduction products, and the electrons released from the oxidation of the aldehyde to the carboxylic acid were only partly accounted for by the reductive reactions identified in this study. This dismutation reaction is at least formally comparable to the situation in *A. woodii*, whereby an increased growth yield was observed in the presence of added 3,4-dihydroxycinnamic acid, which was reduced to the dihydro compound (17). Although our media contained sulfate and possibly, therefore, anaerobic sulfate-reducing bacteria, our consortia were apparently not methanogenic. Therefore, the reactions observed probably bear only a distant relation to hydrogen transfer reactions, which are well documented for anaerobic consortia (20).

The kinetic experiments were used to define the major metabolic processes and the sequence in which they were carried out. At the same time, GC-MS analysis revealed the

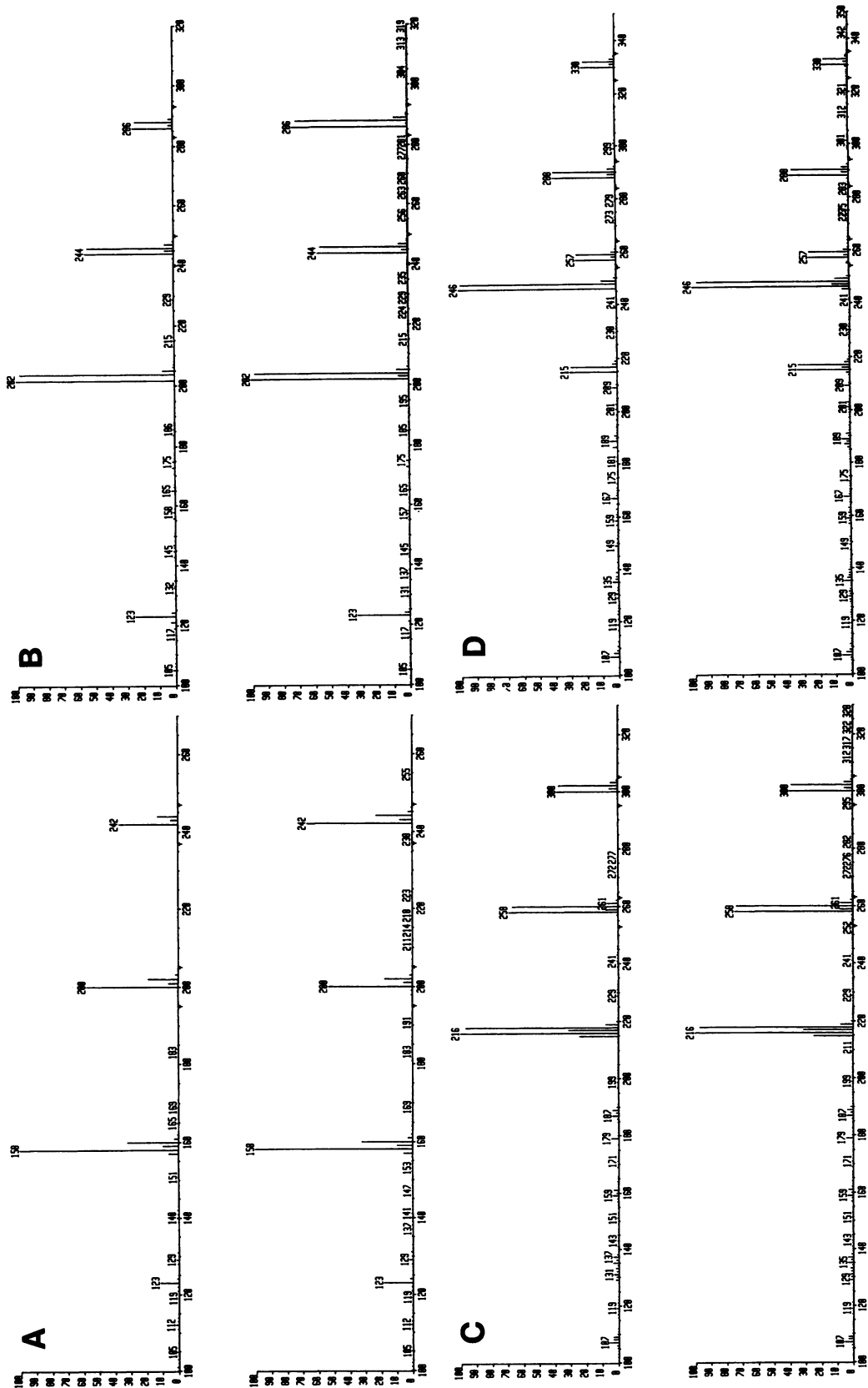


FIG. 4. MS comparison of derivatized metabolites (upper panels) produced by consortium 623 from 5-chlorovanillin (A) and 5-bromovanillin (B to D) with standard compounds (lower panels). (A) 4-Chloro-5-methylcatechol; (B) 4-bromo-5-methylcatechol; (C) 3-bromo-4,5-dihydroxybenzoic acid; (D) 3-bromo-4,5-dihydroxybenzoic acid.

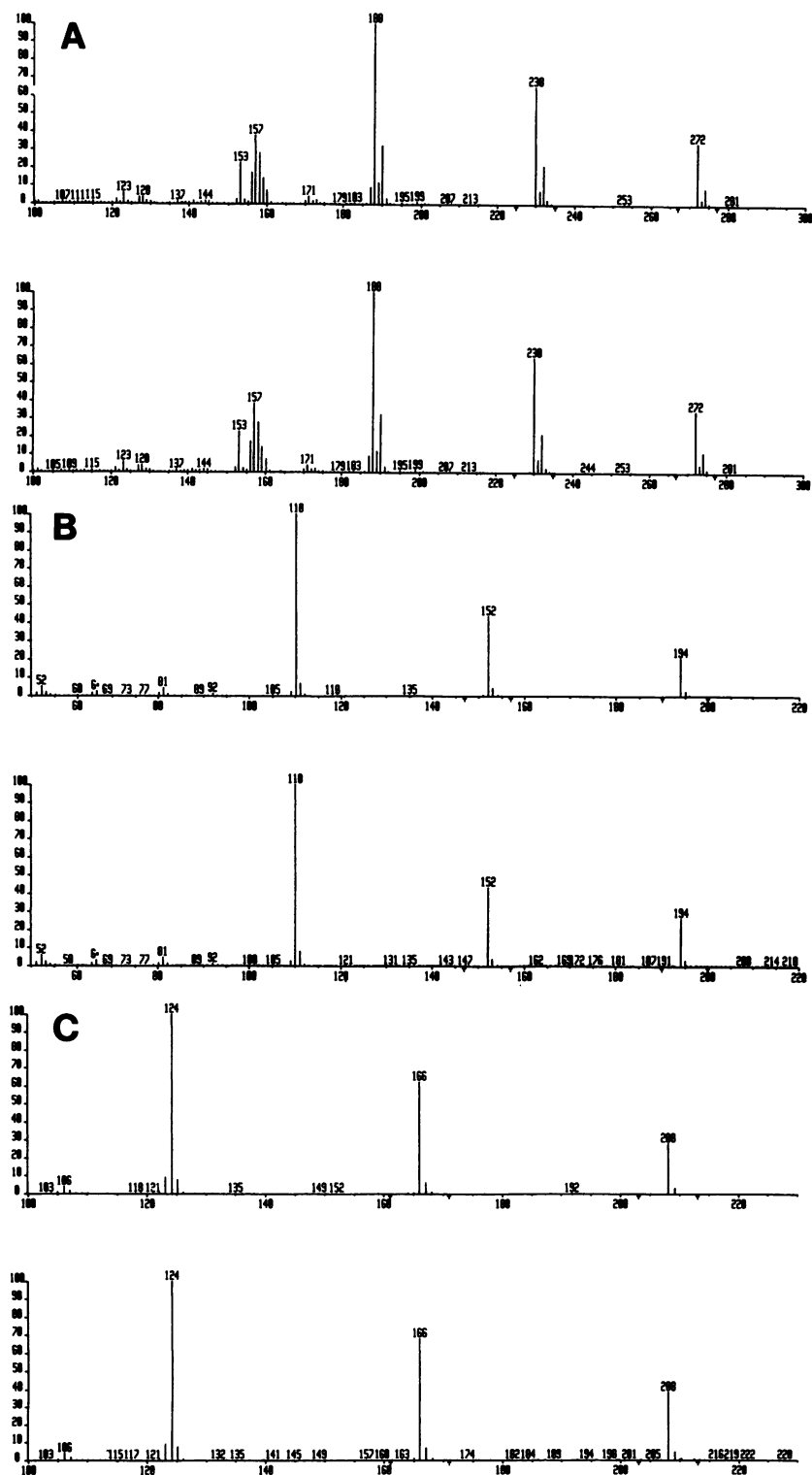


FIG. 5. MS comparison of derivatized metabolites (upper panels) produced by consortium 688 from 5-chlorovanillin (A) and 5-bromovanillin (B and C) with standard compounds (lower panels). (A) 3-Chloro-4,5-dihydroxybenzyl alcohol; (B) catechol; (C) 4-methylcatechol.

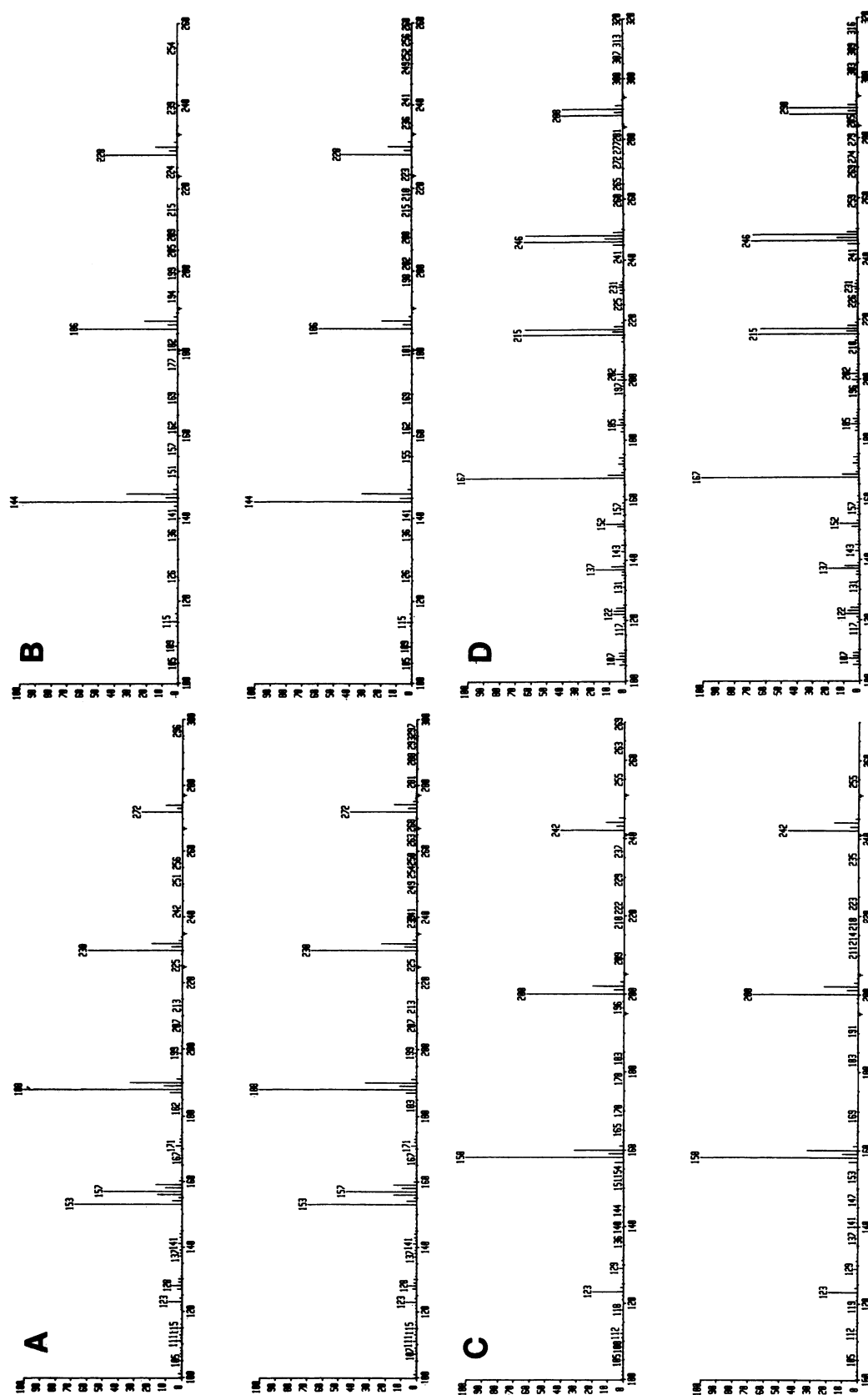


FIG. 6. MS comparison of derivatized metabolites (upper panels) produced by consortium 688 from 6-chlorovanillin (A to C) and 6-bromovanillin (D) with standard compounds (lower panels). (A) 2-Chloro-4,5-dihydroxybenzyl alcohol; (B) 4-chloro-5-methylcatechol; (C) 4-hydroxy-5-methoxybenzyl alcohol; (D) 2-bromo-4-hydroxy-5-methoxybenzyl alcohol.

presence of low but significant concentrations of other metabolites, which have not been quantified in the same way. These data are presented in the form of ratios with respect to major metabolites defined in the kinetic experiments. In addition, ratios are presented for experiments with other consortia in which kinetic experiments were not carried out. It is important to appreciate that all these ratios should be regarded as merely orders of magnitude, since they are certainly dependent on the metabolic state of the consortium at the time when the samples were removed.

The kinetic experiments revealed that production of these reduced metabolites was correlated with metabolism of the initial substrates and, importantly, that formation of reduced products was not one of the initial reactions and never preceded formation of the carboxylic acids. We therefore believe that oxidation of the aldehyde to the carboxylic acid and reduction to the benzyl alcohol were metabolically related. This dismutation is formally analogous to a chemically mediated Cannizzaro reaction.

The demethylated carboxylic acids were generally decarboxylated by consortium 688 to catechols (from the vanillins) and to phenols (from the hydroxybenzaldehydes). Reductive dehalogenation was observed only with 5-bromovanillin, 3-bromo-4-hydroxybenzaldehyde, and 3,5-dibromo-4-hydroxybenzaldehyde and not with any of the chloro compounds examined here.

The anaerobic transformation of aromatic aldehydes appears seldom to have been explored. However, the dismutation of an aldehyde to the corresponding carboxylic acid and benzyl alcohol revealed in this study is clearly analogous to the established transformation of 3-indolylacetaldehyde by ruminal bacteria to 3-indolylacetic acid and 3-indolyl-2-ethanol (21). On the other hand, we are not aware of any previous report of the reduction of an aldehyde to a methyl group. In cases when a glycine residue attached to an aromatic ring is transformed to a methyl group—for example, by certain clostridia (6, 14) or by a mixed ruminal microflora (21)—a methylene group already exists in the substrate. The additional hydrogen in the methyl group is produced by decarboxylation.

The outcome of the reductive processes depends critically on the chemical structure of the aldehyde. We have shown that reduction of the aldehyde group to a hydroxymethyl group occurred with all the substrates (except 5-bromovanillin and 3-bromo-4-hydroxybenzaldehyde) examined and in consortia with various sources and enrichment histories. It was also evident that the extent of this reduction depends on the structure of the aldehyde, since a significantly larger amount of the benzyl alcohol was formed from 3-chloro-4-hydroxybenzaldehyde than from either of the 3,5-dihalogenated compounds. We therefore propose that this reductive transformation may be a widespread and significant, if not major, reaction of halogenated aromatic aldehydes. On the other hand, reduction to a methyl group was apparently a major reaction only in derivatives of halogenated vanillins and, significantly, was not observed with 6-bromovanillin, for which reduction proceeded only to the benzyl alcohol. On the basis of the limited evidence, we suggest a correlation between bacterial growth, the presence of a metabolizable methoxy group, and reduction of the aldehyde to a methyl group.

The nature and position of the halogen substituent exerted a profound influence on the metabolism of the substrate. Although there was essentially no difference in the behavior of 3,5-dichloro-4-hydroxybenzaldehydes and 3,5-dibromo-4-hydroxybenzaldehydes, this was not the case for the halo-

genated vanillins and the monohalogenated hydroxybenzaldehydes: 5-bromovanillin and 3-bromo-4-hydroxybenzaldehyde were debrominated, whereas dehalogenation was never observed with the corresponding chloro compounds. In addition, the position of the halogen in the vanillins had a marked effect on their metabolism: reduction to the benzyl alcohols could be demonstrated for both 6-chlorovanillin and 6-bromovanillin, although ultimate reduction of the aldehyde to a methyl group was observed only with the 6-chloro compound.

From the accumulated evidence, we suggest that reductive reactions of halogenated aromatic aldehydes carrying hydroxy and/or methoxy substituents might be ubiquitous minor pathways in the anaerobic transformation of these compounds. The data demonstrate, however, only the possibility of these reactions: it is important to emphasize that extrapolation of the results of our laboratory experiments to environmental situations obligatorily requires that a number of important factors be taken into consideration. Among these are (i) the extent to which the transformations revealed are carried out during growth with substrates naturally available in anaerobic sediments and (ii) the significance of substrate concentration in determining the outcome of these reactions. In this investigation, substrate concentrations were used which would seldom be found in the natural environment. A previous study of aerobic processes (1) clearly indicated the fundamental differences which may be revealed by using substrate concentrations in the environmentally more plausible range of tenths of a milligram per liter.

This study showed that monochlorovanillins may be reduced to benzyl alcohols and methylcatechols, even though these were not the major metabolites. Although we have not unequivocally determined the fate of these compounds under the experimental conditions used here, this study would suggest that these reduced metabolites might be recoverable from contaminated sediment samples, provided that appropriate extraction methods were used.

In all of the laboratory experiments on anaerobic transformations of halogenated guaiacols and vanillins (10), catechols appear to be the ultimate metabolites. In addition, chlorinated catechols have consistently been recovered from sediment samples collected in the vicinity of bleachery discharge (Remberger et al., in press). We therefore conclude provisionally that catechols are relatively persistent compounds, although anaerobic degradation of catechol itself has been demonstrated (16).

This study further substantiated our view (11) that in hazard evaluations of a compound discharged into the aquatic environment, it is not sufficient to take into account only the original substance: due cognizance must be taken of the occurrence, potential persistence, and toxicity of metabolites formed under environmental conditions.

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